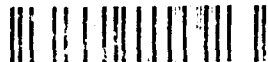


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Joel L. Susman, Ph. D.

CONTRACTING ORGANIZATION: Weizmann Institute of Science  
Departments of Structural Chemistry and  
Neurobiology  
Rehovot 76100, Israel

REPORT DATE: October 1, 1990

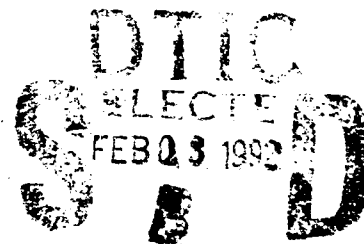
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<p>The objective of this project is the determination of the three-dimensional structure of acetylcholinesterase (AChE) from electric organ tissue of <i>Torpedo californica</i>, with the intention of elucidating the detailed topography of its catalytic site.</p> <p>In the period covered by this report, work was carried on the dimeric form of AChE from the above source, purified by affinity chromatography subsequent to solubilization with phosphatidylinositol-specific phospholipase C, which contains two catalytic subunits (each of <math>M_r = 65,000</math>) linked by a single disulfide bond.</p> <p>Crystals were obtained from this preparation by vapor diffusion, using ammonium sulfate as the precipitant. These crystals belong to space group <math>P3_121</math> with unit cell dimensions <math>a=b=110.9 \text{ \AA}</math> and <math>c=136.9 \text{ \AA}</math>.</p> <p>Native X-ray data sets were collected on a Siemens/Xentronics Area Detector to <math>2.8 \text{ \AA}</math></p>					
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resolution. A search for heavy-atom derivatives was conducted using six different heavy-atom compounds under a variety of soaking conditions. Soaking with 2mM  $\text{UO}_2(\text{NO}_3)_2$  in a phosphate-free mother liquor for 8 days, yielded an isomorphous heavy-atom derivative. Based on difference Patterson maps, it was possible to find two major and two minor sites for this derivative. A second heavy-atom derivative, obtained by soaking with  $\text{HgAc}_2$  (15mM) for 9 days, contained two mercury atoms.

A number of sulfhydryl reagents inhibit *Torpedo californica* AChE with pseudo-first-order kinetics, the best of those examined being *N*-ethylmaleimide and the organomercurial *p*-chloromercurisulfonic acid. Inhibition can be retarded by quaternary ligands which bind at either the catalytic or peripheral anionic binding sites. Colorometric titration with one of the inhibitory sulfhydryl reagents, 5,5'-dithiobis(2-nitrobenzoic acid), reveals a single thiol group per catalytic subunit; our data suggest that inhibition is achieved by reaction with a single free thiol group of Cys-231, which may offer a site for insertion of a covalently linked heavy-atom derivative.

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*A. Selman* *Jan 8, 1992*  
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## SUMMARY

The objective of this project is the determination of the three-dimensional structure of acetylcholinesterase (AChE) from electric organ tissue of *Torpedo californica*, with the intention of elucidating the detailed topography of its catalytic site.

In the period covered by this report, work was carried on the dimeric form of AChE from the above source, purified by affinity chromatography subsequent to solubilization with phosphatidylinositol-specific phospholipase C, which contains two catalytic subunits (each of  $M_r = 65,000$ ) linked by a single disulfide bond.

Crystals were obtained from this preparation by vapor diffusion, using ammonium sulfate as the precipitant. These crystals belong to space group  $P3_121$  with unit cell dimensions  $a=b=110.9 \text{ \AA}$  and  $c=136.9 \text{ \AA}$ .

Native X-ray data sets were collected on a Siemens/Xentronics Area Detector to  $2.8 \text{ \AA}$  resolution. A search for heavy-atom derivatives was conducted using six different heavy-atom compounds under a variety of soaking conditions. Soaking with  $2\text{mM UO}_2(\text{NO}_3)_2$ , in a phosphate-free mother liquor for 8 days, yielded an isomorphous heavy-atom derivative. Based on difference Patterson maps, it was possible to find two major and two minor sites for this derivative. A second heavy-atom derivative, obtained by soaking with  $\text{HgAc}_2$  ( $15\text{mM}$ ) for 9 days, contained two mercury atoms.

A number of sulfhydryl reagents inhibit *Torpedo californica* AChE with pseudo-first-order kinetics, the best of those examined being *N*-ethylmaleimide and the organomercurial *p*-chloromercurisulfonic acid. Inhibition can be retarded by quaternary ligands which bind at either the catalytic or peripheral anionic binding sites. Colorimetric titration with one of the inhibitory sulfhydryl reagents, 5,5'-dithiobis(2-nitrobenzoic acid), reveals a single thiol group per catalytic subunit; our data suggest that inhibition is achieved by reaction with a single free thiol group of Cys-231, which may offer a site for insertion of a covalently linked heavy-atom derivative.





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## OBJECTIVES

The long-term objective of this project is the elucidation, by X-ray crystallography, of the three-dimensional structure of acetylcholinesterase from *Torpedo californica*. An interim objective, to which our major efforts were devoted during the first year, was to obtain one or more isomorphous heavy-atom derivatives, a crucial step in the structure determination.



## BACKGROUND

The principal biological role of acetylcholinesterase (AChE) is the termination of impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (Barnard, 1974). AChE is, accordingly, characterized by a remarkably high specific activity, especially for a serine hydrolase (Quinn, 1987), functioning at a rate approaching that of a diffusion-controlled reaction (Bazelyansky *et al.*, 1986). The powerful acute toxicity of organophosphate and carbamate poisons is attributed primarily to their action as potent covalent inhibitors of AChE (Koelle, 1963; Aldridge and Reiner, 1972). Elucidation of the three-dimensional structure of AChE is thus of fundamental interest in understanding its remarkable catalytic efficacy, and of paramount importance in the development of therapeutic approaches to organophosphorus poisoning.

The electric organs of the electric fish *Torpedo* and *Electrophorus* provide rich sources of AChE (Nachmansohn and Neumann, 1975). These forms are structurally and functionally homologous to the corresponding forms in vertebrate nerve and muscle (Massoulié and Bon, 1982); highly purified preparations from electric organ tissue have, therefore, provided much of our knowledge concerning both the structure and the function of AChE (Rosenberry, 1975; Quinn, 1987; Silman and Futerman, 1987). AChE from *Torpedo californica* has been cloned (Schumacher *et al.*, 1985; Sikorav *et al.*, 1987), and both its primary sequence and arrangement of disulfide bridges are known (MacPhee-Quigley *et al.*, 1985, 1986). In *Torpedo* electric organ, one of the principal forms of AChE is a disulfide-linked catalytic subunit dimer belonging to a recently described class of membrane proteins which are linked to the plasma membrane via covalently attached phosphatidylinositol (PI) (Low *et al.*, 1986). In such proteins, the PI is attached at the carboxyl-terminus of the polypeptide chain through an intervening oligosaccharide sequence which is added posttranslationally (Ferguson and Williams, 1988), and the diglyceride moiety of the PI serves as the hydrophobic anchor. PI-anchored proteins can often be solubilized by a PI-specific phospholipase C (PIPLC) of bacterial origin (Low *et al.*, 1986), and this is also the case for the *Torpedo* AChE dimer.

The 11 S tetrameric form of AChE from *Electrophorus electricus* was first crystallized by Leuzinger and Baker (1967), and preliminary characterization of crystals obtained from this form was subsequently reported (Chothia and Leuzinger, 1975; Schrag *et al.*, 1988). Sequence information concerning *Electrophorus* AChE is not yet available. Furthermore, the 11 S tetramer possesses proteolytic "nick" sites due to its mode of preparation (for a discussion see Anglister and Silman, 1978), and the best crystal form reported by Schrag *et al.* (1988) diffracts asymmetrically

to between 3.5 and 6 Å, depending upon direction. Therefore, we selected the dimer from *Torpedo* electric organ as a more suitable candidate for crystallization and for subsequent structural studies. We adopted, for this purpose, a novel and mild purification procedure involving solubilization with PIPLC, followed by affinity chromatography, to yield a highly purified water-soluble preparation of the *Torpedo* dimer which differed from the native enzyme only in the removal of the diglyceride moiety of the membrane anchor (Futerman *et al.*, 1985; Sussman *et al.*, 1988).

As we already reported (Sussman *et al.*, 1988), crystals of the *Torpedo* dimer were obtained initially from polyethylene glycol (PEG) 200 at pH 6.0. Bipyrarnidal crystals were obtained which diffracted to 2 Å resolution, but displayed substantial decay in the X-ray beam. These crystals were found to be orthorhombic, belonging to space group P222<sub>1</sub>. Subsequently, crystals were also obtained from 60% ammonium sulfate-0.1 M phosphate, pH 7.0. These crystals were trigonal, grew to as long as 1mm in their longest dimension, diffracted to 2.8 Å resolution, and lost as little as 10% of their diffracting power in the X-ray beam. The advantage of crystals grown from salt solution over those grown from organic solutions is that the former are more likely to withstand cooling to cryogenic temperatures, at which there is virtually no decay of diffracting power with time in the X-ray beam. Indeed, we found this to be the case for the AChE crystals which were obtained from ammonium sulfate (Sussman *et al.*, 1989).

## TECHNICAL APPROACH

### 1. Enzyme purification and characterization

The dimeric form of AChE was purified in water-soluble form, essentially by the procedure reported earlier (Futerman *et al.*, 1985; Sussman *et al.*, 1988), which involves solubilization with PI-specific phospholipase C (PIPLC) followed by affinity chromatography. In the procedure currently employed, PIPLC purified from *Staphylococcus aureus* has been replaced by PIPLC purified from *Bacillus thuringiensis* (Low *et al.*, 1988) or, more recently, by PIPLC produced by *Bacillus subtilis* transfected with the PIPLC gene from *Bacillus thuringiensis* (Henner *et al.*, 1988). PIPLC from *Bacillus thuringiensis* has been reported to have a much higher specific activity than *Staphylococcus aureus* PIPLC for many of its substrates (Low *et al.*, 1988). We have found that this holds true also for the solubilization of the PI-anchored AChE dimer of *Torpedo* electric organ, as little as 0.15 U/ml of homogenate (corresponding to ca. 0.1 µg/ml of enzyme protein) yielding quantitative release of the AChE dimer under the incubation conditions employed. The enzyme produced by transfection into *Bacillus subtilis* appeared to be equally effective.

The purity of the AChE preparations obtained was assessed, prior to their use for crystallization or for biochemical studies, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentrations were determined either from the absorption at 280 nm, using  $A^{1\%}_{280} = 17.5$  (Taylor *et al.*, 1974), or by the procedure of Bradford (1976). Specific activity was determined both by the radiometric procedure of Johnson and Russell (1975) and by the colorimetric procedure of Ellman *et al.* (1961), using acetylthiocholine as substrate. Both these enzymic assays were also employed in studies on the inactivation of the *Torpedo* dimer by thiol reagents.

### 2. Crystallization

Studying and understanding the mechanism and action of enzymes requires the knowledge of their three-dimensional structures which for large molecules can, at present, be obtained only by single crystal X-ray diffraction methods. Great advances have been made in the field of protein crystallography in recent years (Wyckoff *et al.*, 1985). The major difficulty *en route* to solving the structure is the stage of crystal growing. A suitable crystal must diffract to atomic resolution, withstand damage caused by X-ray irradiation for a reasonable period of time, and grow to a size which yields a measurable diffraction pattern.

Although no sure recipe exists for protein crystal growth, a great deal of empirical data exist (Gilliland, 1990). In order to screen thousands of different crystallization conditions with

efficient use of the purified enzyme preparation, we used the hanging drop vapor diffusion technique (McPherson, 1985) at both 19°C and 4°C. Using drops of between 5 and 10  $\mu$ l and a stock AChE solution of protein concentration 10-15 mg/ml, we were able to survey 50-100 sets of conditions using only 1 mg of protein.

### 3. Heavy-atom derivatives

In order to determine the three-dimensional structure of a protein, it is necessary to solve the so called "phase problem." This can be done either by molecular replacement (Rossmann, 1972) or by multiple isomorphous replacements (MIR) (Blundell and Johnson, 1976). Since, thus far, there are no closely related cholinesterases for which three-dimensional structures are available and which could thus serve as starting models for molecular replacement for AChE, it was necessary for us to obtain phases *ab initio*, by the MIR method; this, in turn, required the preparation of suitable isomorphous heavy-atom derivatives.

Screening for heavy atom derivatives was performed by diffusion of candidate heavy atom compounds into native crystals. This screening was carried out both in the mother liquor in which the native crystals were grown, **M0** (60% ammonium-sulfate, 0.36 mM Na<sup>+</sup>, K<sup>+</sup>-phosphate buffer, pH 7.0; see "Results"), and also under different conditions, primarily to have a phosphate-free mother liquor. This was achieved by transferring the crystals to either **M1** (65% ammonium-sulfate, 100 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.9) or **M2** (65% ammonium-sulfate, 100 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), pH 7.0).

### 4. X-ray-data collection

Collection of X-ray data from crystals of large biological macromolecules is often quite difficult. Due to their size, the unit cells of such molecules are very large. In order to obtain sufficient data to determine their structures at atomic resolution, it is necessary to record tens of thousands of X-ray reflections. Usually, due to the irregular shape of their molecular surfaces, macromolecules pack in the crystal in a manner such that a large proportion of its volume is solvent. As a result, many macromolecular crystals diffract X-rays rather poorly. Therefore, in order to obtain X-ray data of high enough accuracy it is necessary to measure each reflection for a longer time than is required for strongly diffracting crystals. Measurement of a full data set from crystals of a multimeric enzyme may thus take a long time (days or even weeks). Since such crystals are often sensitive to X-ray irradiation, their diffracting power sometimes decays rapidly after irradiation has commenced (often within days or even within hours). In order to collect large



sets of X-ray data for such sensitive crystals, one needs either extremely fast collection methods, a way to prolong the useful lifetime of the crystal, or, ideally, a combination of the two.

*a. Rapid X-ray -data collection for macromolecules*

In our laboratory, accurate single-crystal X-ray data are routinely collected at room temperature on a Rigaku Rotating Anode Diffractometer (AFC5-R). Unfortunately, collection of a complete set of X-ray data to  $\sim 2.8$  Å resolution for the crystals of *Torpedo* AChE would take several weeks, well beyond the lifetime of these crystals in the X-ray beam ( $\sim 24$  hours). Use of the Siemens/Xentronics Area Detector, mounted on a rotating anode, permits collection of such a data set within 12-24 hours, depending on crystal size. We are, therefore, routinely employing such an apparatus for data collection both from native crystals and from putative heavy-atom derivatives. The Siemens/Xentronics Area Detector is mounted on a Rigaku RU300 rotating-anode generator operating at 40 kV/250 mA, with a graphite monochromator and a 0.5x10 mm filament, takeoff angle 6°. Three different plate-to-crystal distances are used: 150 mm, 158 mm, and 170 mm. Data are collected in a series of frames, each recording a small oscillation angle ( $\omega$ ) of the crystal (usually 0.25°). The collection time required is 100-300 seconds per frame. In order to obtain three-dimensional X-ray data from these frames, they are processed by the XENGEN (Howard *et al.*, 1987) or XDS (Kabsch, 1988) computer programs. At present, with our current computing power, the frame-processing procedure actually takes somewhat longer than collection of the frames themselves.

*b. Cryogenic X-ray-data collection*

A procedure developed in our laboratory during the past few years with great success (Hope, Frolov and Sussman, 1987) results in almost indefinite prolongation of the lifetime of many crystals in the X-ray beam. This procedure involves shock-cooling the crystal to an extremely low temperature,  $-150^{\circ}\text{C}$  (Hope, 1988), following by collection of X-ray data at the same temperature. This has been achieved, for the first time in a routine manner, in our laboratory, by grafting a low-temperature attachment (constructed "in house") onto a Rigaku AFC5-R rotating anode diffractometer (Hope, Frolov and Sussman, 1987). This permits cooling of a crystal by a stream of boiled  $\text{N}_2$  gas during data collection without impeding the movements of the diffractometer. We have already shown for over a dozen different crystals of proteins and nucleic acids (Sussman, 1988; Joshua-Tor *et al.*, 1988; Harel *et al.*, 1990) that crystal life is prolonged indefinitely under these experimental conditions. It is thus possible to collect an entire data set from a single crystal of AChE on a rotating anode diffractometer. Although the time for high

resolution data collection is still rather long, the procedure obviates the need to scale data from several crystals and thus yields very accurate data.

Although the principle of cryogenic X-ray-data collection is simple, it is, in fact, rather difficult in practice. Several key technical features, developed in our laboratory, are described below:

*i. Cryoprotectants.* In order to maintain the crystallographic integrity of the crystal during the mounting procedure it is first coated by an inert oil (protectant), which serves as a protective shield and, thus, prevents drying and/or precipitation of salt during transfer of the crystal to the stream of cold N<sub>2</sub>. In addition, the mother liquor will freeze on cooling; it is, therefore, desirable to remove as much of it as possible from the crystal surface. The cryoprotectant routinely used for work on AChE is the resin part of Duro Master Mend Epoxy (Loctite Corporation, Stock No. QM-50).

*ii. Crystal-transfer from mother liquor to protectant.* A procedure for transferring a crystal from its growth solution into an appropriate hydrophobic environment of the cryoprotectant is illustrated in Figure 1. The crystal is moved, via a short solvent channel, from the mother liquor to the protectant by use of a suitable tool (e.g., a thin glass fiber); it is then carefully "washed" simply by moving it around. This procedure will remove the mother liquor adhering to the crystal. Since the layer of protectant should be as thin as possible, the crystal, provided that it is sufficiently robust, is placed on a portion of the siliconized cover slip which is free of both protectant and mother liquor, and carefully moved backwards and forwards. Figure 1d shows the final mounting position of the crystal.

*iii. Crystal-mounting and "flush"-freezing.* As in small molecule crystallography, a technique for crystal mounting, using a glass fiber of appropriate length and thickness, is employed (a capillary is inappropriate, since it can act as an insulator in the cooling process). The glass fiber is attached to the copper mounting rod, and its tip, to which the crystal will adhere, is covered evenly by high vacuum-grease. The high vacuum-grease which we employ is produced by CVC Products, Inc. (m.p. 120°C, vapor pressure to 106 torr at 25°C, P/N 269352-0012, made in USA).

Transfer of the mounted crystal into the stream of cold N<sub>2</sub> should be carried out as quickly as possible to prevent formation of ice crystals. This is achieved by temporary blocking of the laminar stream (e.g., by holding a stiff paper across the nozzle), while the crystal is being brought

to its final position on the measuring device, followed by rapid release of the block, with resultant "flash"-freezing of the crystal, as desired.

During the past year, we have constructed a low-temperature device specifically designed for use on the Xentronics/Siemens Area Detector. This powerful tool now combines the rapid collection features of the area detector with the long life and accuracy of the low-temperature device. For native data sets, this setup is now being employed routinely. However, for screening of putative heavy-atom derivatives and comparison with native data, the area detector is still operated at room temperature, since shock-cooling sometimes alters the unit cell dimensions of the crystal more than is acceptable for MIR. We anticipate, however, that use of the area detector at cryogenic temperatures will be invaluable for studying the high-resolution structure of the native enzyme, as well as of enzyme-substrate and enzyme-inhibitor complexes and conjugates.

## **5. Crystallographic-computing**

The scaling and merging of the X-ray data that we have collected from the AChE crystals is routinely done on the Weizmann Institute Computer Center's Micro-VAX 3600, employing the CCP4 suite of programs (obtained from the Daresbury Laboratory, U.K). In addition, all of the computing involved in the search for heavy-atom derivatives, refinement of their positions, and calculation of Fourier maps is also being done with the CCP4 programs on this VAX.

## RESULTS and DISCUSSION

### 1. Crystal characterization

Crystallization conditions for the crystal form of *Torpedo* AChE obtained from ammonium sulfate, as described above, were refined to permit production of crystals in a routine manner. The crystals are grown by standard vapor-diffusion techniques, at 19°C, in hanging drops, from a stock solution of protein concentration ~13 mg/ml. The precipitant used is 60% saturated ammonium sulfate in 0.36 mM Na<sup>+</sup>, K<sup>+</sup>-phosphate buffer, pH 7.0. The ammonium-sulfate concentration employed may vary slightly from batch to batch of enzyme. Crystals grow as flattened pyramids, up to ~1 mm in their longest and ~0.5 mm in their shortest dimension, over a period of several weeks after nucleation (Figure 2). Initial X-ray studies at low temperature (90°K), using a Rigaku AFC5-R rotating-anode diffractometer operated at 10 kW, revealed that these crystals belong to either space group P3<sub>1</sub>21 or P3<sub>1</sub>12, with cell dimensions a=b=110.9 Å and c=136.9 Å. The unit-cell dimensions correspond to a volume of ~1.5x10<sup>6</sup> Å<sup>3</sup>, which gives a value for V<sub>m</sub> (volume/unit mass) of 1.9 Å<sup>3</sup>/dalton, assuming that there is one dimer per asymmetric unit. The ratio of the volume of the asymmetric unit to the molecular weight of the dimer (~130 kD) is within the range outlined by Matthews (1968). This AChE dimer is believed to consist of two identical catalytic subunits linked by a single disulfide bridge (Silman and Futerman, 1987). The crystals diffract to about 2.8 Å resolution and are stable for about 2 days in the X-ray beam at room temperature. Due to the large size of the unit cell, it is difficult, or impossible, to collect complete data sets from single crystals on a standard diffractometer at room temperature. X-ray data were, therefore, collected on the Siemens/Xentronics Area Detector. A typical 0.25° frame, obtained on the Area Detector for a native crystal, is shown in Figure 3.

In order to distinguish, unambiguously, between the two space groups, P3<sub>1</sub>21 and P3<sub>1</sub>12, with their Laue symmetry P3m1 and P31m, respectively, one needs to examine symmetry-related reflections. This was done by examining zonal hk0 and hk1 precession photographs. The hk0 zone, showing hexagonal symmetry, does not allow one to distinguish between a 2-fold axis and a mirror-plane, either of which is located between the axes of the primitive cell in P3<sub>1</sub>21 or P3<sub>1</sub>12, respectively. A 2-fold axis, present in the space group P3<sub>1</sub>21, will, however, vanish in the hk1 zone, whereas a mirror-plane, present in the space group P3<sub>1</sub>12, will remain, a fact which allows one to distinguish between the two space groups. The hk1 zone clearly indicates the loss of symmetry between the axes of the primitive cell, indicating that the symmetry element in the hk0 zone is a 2-fold axis. This confirms the space group as P3<sub>1</sub>21 or its enantiomorph P3<sub>2</sub>21.

## 2. Data collection and data-processing

Data were collected on a Siemens/Xentronics Area Detector, at room temperature, as described above.

### a. Native data

A first data set to 3.0 Å resolution (AChE\_1RT) was collected for a native AChE crystal with an overall  $R_{\text{sym}}$  of 0.106, ranging from 0.052 (for the lowest shell of data, between  $\infty$  and 5.4 Å), to a value of 0.40 (for the data between 3.2 and 3.0 Å). As there are two different settings for the space group  $P3_121$ , this data set defined the reference system (hkl). Crystallographic data, measured in the second setting, were transformed to the reference system in the following way:  $h_{\text{new}} = +k$ ,  $k_{\text{new}} = -h$ ,  $l_{\text{new}} = l$ .

Since we intended to screen for heavy-atom derivatives under conditions different from the original precipitating reservoir and mother liquor, it was necessary also to collect native data sets under these different conditions. The conditions were selected to produce a phosphate-free environment. Thus, native data sets were collected not only in the original mother liquor, **M0**, at pH 7.0, but also in the phosphate-free media, **M1** (pH 6.9) and **M2** (pH 7.0), as described above under Technical Approach. The crystals were observed to be stable under all three sets of conditions used, showing only some minor splits on their surface. Unit cell dimensions for crystals in **M1** and **M2** changed by less than 1% from those obtained in **M0**, and the difference Patterson's (calculated for control), using AChE\_1RT as the native data, were featureless. Table 1 gives a summary of the pertinent information for the three native data sets.

As was discussed above (see X-ray Data Collection), shock-cooling sometimes alters the unit cell dimensions of the crystal more than is acceptable for MIR, therefore the above X-ray data were collected at room temperature. We observed that at room temperature, the diffraction intensities of AChE crystals fell off by about 10% in 24 hours, which we set as an upper limit of decay. In contrast, at 90K, the crystals survived virtually indefinitely with no decay, i.e. several weeks, and that is why we plan to collect most of our high resolution native data, for the purpose of detailed crystallographic refinement, at cryogenic temperatures.

### b. Screening of heavy-atom compounds

As mentioned above, heavy-atom screening was performed by soaking native crystals maintained in buffers **M0**, **M1**, or **M2** at the heavy-atom concentrations listed in Table 2. It should be noted, that after the crystals were transferred to buffers **M1** or **M2**, they were permitted

to equilibrate for at about 24 hours, before the heavy atom screening in these new buffers was begun. The heavy-atom compounds tested included organomercurials, which it was hoped would interact with the single free sulfhydryl group on Cys-231 in AChE of *Torpedo californica* (Schumacher *et al.*, 1985; MacPhee-Quigley *et al.*, 1986) and a number of commonly employed non-specific heavy-atom compounds. For each potential heavy atom derivative, data sets were collected from a single crystal alone.

Three-dimensional Patterson maps were calculated for each data set, using  $\|FPH| - |FP|\|^2$  as coefficients, where FP corresponds to the structure factor amplitudes obtained from the native crystal soaked in the corresponding mother liquor. As can be seen from Table 2, of the six heavy-atom compounds examined, two, namely  $UO_2(NO_3)_2$  and  $HgAc_2$ , have already yielded promising heavy-atom derivatives.

The best uranyl derivative, UO1, was obtained upon soaking the crystal for 8 days in 2mM  $UO_2(NO_3)_2$  dissolved in M1. The difference Patterson map for this uranyl nitrate derivative, calculated at 5 Å resolution, is interpretable in terms of two major uranyl sites located at general positions in the asymmetric unit of the unit cell. These sites have been confirmed by the anomalous Patterson, as well as by both the automatic Patterson-solution routine and the Direct Methods of SHELXS-86. Figure 4 shows the Harker section at  $w=1/3$  of the difference and anomalous Pattersons. Two minor heavy-atom sites were detected in *double-difference* Fourier maps. Refinement of all four sites, including anomalous contributions, yielded an  $R_{Cullis}$  of 39.5%, for 15-3.5Å data (where a relatively low  $R_{Cullis}$ , such as this 39.5% implies both a good derivative, as well as that the refinement went well). The refined heavy-atom parameters obtained are:

	Site 1	Site 2	Site 3	Site 4
Isomorphous occupancy	1.05	0.63	0.15	0.15
Anomalous occupancy	1.43	0.77	0.17	0.16
x	0.15	0.43	0.20	0.80
y	0.48	0.95	0.33	0.23
z	0.17	0.07	0.10	0.10
$B(\text{\AA})^2$	29.5	49.5	22.0	54.3

Phasing to 3.5 Å resolution, taking the isomorphous and anomalous contributions of the four sites, gives a figure-of-merit of 0.80 (where the higher the figure-of-merit implies overall better phases).

The quality of the uranyl nitrate derivative which we have obtained is such that it permits screening for other putative heavy-atom derivatives by means of difference Fourier maps phased on the uranyl nitrate, which are much easier to interpret than difference Patterson maps. The phases generated by the uranyl derivative were used to calculate a difference Fourier map of the HgAc<sub>2</sub> derivative HGAC4. This map yielded two sites for the Hg derivative which can be readily seen in the two sections of the difference Fourier map shown in Figure 5. The two sites, including anomalous data, were refined to yield an  $R_{\text{Cullis}}$  of 55.7%. The refined heavy atom parameters obtained are:

	Site 1	Site 2
Isomorphous occupancy	0.75	0.42
Anomalous occupancy	1.91	1.10
x	0.21	0.45
y	0.61	0.30
z	0.08	0.02
B(Å) <sup>2</sup>	14.4	22.2

Phasing to 2.8 Å resolution, taking the isomorphous and anomalous contributions of the two sites, gives a figure-of-merit of 0.74. We are currently actively engaged in screening for additional derivatives in this manner.

### 3. Modification of *Torpedo* AChE by thiol agents

A valuable approach to the determination of the three-dimensional structure of proteins involves chemical modification with group-specific agents containing heavy atoms. Suitable selection of the reagent and of the reaction conditions may thus permit insertion of heavy atoms at known points within the amino acid sequence. Although, in general, AChE does not appear to display the characteristic sensitivity of sulfhydryl enzymes to organomercurials and alkylating agents (Mounter and Whittaker, 1953; Karlin, 1967; see also Wilson and Silman, 1977), AChE from *Torpedo californica* is unusual in containing a free sulfhydryl group on Cys-231 (MacPhee-Quigley *et al.*, 1986). Tomlinson and coworkers (Mutus *et al.*, 1983; Tomlinson and Kinsch, 1989) have reported that purified AChE from this source can bind 1 mol of a fluorescent sulfhydryl agent, *S*-mercuri-*N*-dansylcysteine, per mole of catalytic subunits, without affecting

catalytic activity. We decided, therefore, to examine the effect of various organomercurial and other sulfhydryl reagents on *Torpedo californica* AChE.

We were able to show that a repertoire of typical sulfhydryl agents all inhibit purified *Torpedo californica* AChE with pseudo-first-order kinetics (Figure 6). Of these reagents, the most effective is *p*-chloromercurisulfonic acid (PCMS), which at pH 7.0 and room temperature, at 0.1 mM, inhibits with a  $t_{1/2}$  of 51 minutes. Several other reagents, however, including, in particular, *N*-ethylmaleimide, are also quite effective inhibitors (Table 3).

Colorimetric titration of the denatured protein with one of these reagents, 5,5'-dithiobis(2-nitrobenzoic acid), reveals a single thiol group per catalytic subunit, presumably that of Cys-231.

In *Torpedo californica* AChE, Ser-200 is the active-site residue, suggesting that the effect of the thiol reagents may be ascribed to the close proximity of the SH-group of Cys-231 to the active site. Indeed, modification of this group by PCMS precluded any measurable reaction of the organophosphates paraoxon and 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide (MEPQ) with the active site serine, as shown by spectrophotometric monitoring of the release of *p*-nitrophenol and 7-hydroxy-1-methylquinolinium, respectively, for control and modified enzyme.

In keeping with this last observation were experiments which showed that the powerful reversible AChE inhibitor edrophonium dramatically reduces the rate of inhibition by the thiol reagents *N*-ethylmaleimide (NEM) and PCMS (Table 4). In contrast, another such inhibitor, *N*-methylacridinium, has no effect. This may be due to the fact that edrophonium occludes both the anionic and catalytic sites, whereas *N*-methylacridinium reacts only with the active site (for a discussion see Abramson *et al.*, 1989). The fact that three quaternary thiol reagents 4-(*N*-maleimido)phenyltrimethylammonium iodide (MPTA), 4-(*N*-maleimido)- $\alpha$ -benzyltrimethylammonium iodide (MBTA) and bromocethylcholine (BACA), which are known to serve as affinity labels of the nicotinic acetylcholine receptor (Karlin and Winnik, 1968; Ben-Haim *et al.*, 1973), inactivate AChE at rates similar to those displayed by comparable uncharged reagents, also suggests that the thiol of Cys-231 is not in range of the anionic site. A final complication is the observation that ligands which recognize the "peripheral" anionic site, such as propidium and gallamine, also retard the rate of reaction of PCMS and NEM; whether they achieve their effect "allosterically" or directly remains to be established. In fact, an answer to several of the questions raised by the data presented above will lie in the localization of the thiol group, as labelled by a suitable organomercurial, within the three-dimensional structure of the enzyme.



## CONCLUSIONS

The principal achievement of the first year of this contract was to obtain two distinct isomorphous heavy-atom derivatives, namely, a uranyl derivative and a mercuric derivative, of our native *Torpedo* AChE crystals. This is a key step *en route* to determining the three-dimensional structure of acetylcholinesterase. It appears that the PCMBS (thiol) derivative, which specifically labeled Cys 231, did not greatly modify the AChE structure based on its high degree of isomorphism and consequently how good a heavy atom derivative it was. The X-ray data collected from these two heavy atom derivatives, together with our native data, should be enough to obtain MIR phases in order to calculate a 2.8Å electron density map. We feel that it is likely that this map will be of sufficient quality so that we will be able to trace the polypeptide chain of the AChE molecule.



TABLE 1. Native room temperature AChE data

Name	Unit cell parameters a = b c	# of measurements	# of unique reflections	Completeness (%)	Maximum* resolution	R <sub>sym</sub> (I) (%)	R <sub>sym</sub> (F) (%)	R <sub>merge</sub> (F) versus: -IRT (%) -MES (%) -HEPES (%)
AChE-IRT	114.6 138.8	48823	17837	82	3.0 Å	9.7	4.6	7.7 10.1
AChE-MES	113.7 137.9	32183	7717	64	3.6 Å	13.1	5.5	7.7 8.7
AChE-HEPES	113.5 138.4	20515	4821	83	4.6 Å	9.1	4.4	10.1 8.7

\* The maximum resolution to which the crystals diffract pertain to this particular experiment and does not imply that different crystals under the same conditions would not diffract to higher resolutions.

TABLE 2. Heavy atom screening

Compound	Concentration	Soaking time	Mother liquor	Comments	XTAL name	Completeness (%)	Maximum resolution	$R_{\text{merge}}$ - (native) (%)	Comments
PCMBS	2 mM	2 h	MO	5 $\lambda$ added to drop	PC1	81	3.2 Å	8.2 (IRT)	No significant features in Difference Patterson xtal dissolved
PCMBS	2 mM	23 h	M1		PC2(a)	43	3.6 Å	--	xtal slipped in capillary
PCMBS	0.2 mM	23 h	M1		PC2(b)	--	--	--	Noninterpretable Patterson
PCMBS	2 mM	20 h	MO		PC3	66	3.8 Å	22.5 (IRT)	"
$K_2PtCl_4$	0.5 mM	5 h	MO	5 $\lambda$ added to drop	PTCL1	76	3.1 Å	6.7 (IRT)	xtal slipped in capillary
$K_2PtCl_4$	2 mM	4 d	M1		PTCL2	--	--	--	xtal died in X-ray beam
$K_2PtCl_4$	0.2 mM	3 d	MO		PTCL3	--	--	--	"
HgAc <sub>2</sub>	0.2 mM	29 h	MO	5 $\lambda$ added to drop	HGAC1	90	4.4 Å	9.2 (IRT)	No significant features in Difference Patterson
HgAc <sub>2</sub>	2 mM	2 d	M1		HGAC2	87	3.8 Å	8.7 (MES)	"
HgAc <sub>2</sub>	3 mM	7 h	M2	Small cracks	HGAC3	43	4.6 Å	8.0 (HEPES)	Two-site derivative
HgAc <sub>2</sub>	15 mM	9 d	M1		HGAC4	96	2.8 Å	13.1 (MES)	Two major sites
$UO_2(NO_3)_2$	20 mM	2 d	M1	Precipitated with MO	UO1	67	3.8 Å	19.6 (MES)	Two major & two minor sites
$UO_2(NO_3)_2$	2 mM	8 d	M1		UO2	86	2.6 Å	17.7 (MES)	No significant features in Difference Patterson
NaAuCl <sub>4</sub>	1 mM	2 d	MO		AU1	52	2.9 Å	9.4 (IRT)	

**TABLE 3. Kinetic constants for inactivation of AChE by sulfhydryl reagents**

The pseudo-first-order rate constant ( $k_i$ ) for inactivation at the concentration of sulfhydryl reagent employed, and  $t_{1/2}$  is the time required to achieve 50% inhibition of enzymic activity.

Sulfhydryl reagent	Concentration (mM)	$k_i$ (min <sup>-1</sup> )	$t_{1/2}$ (min)
PCMS	0.1	$1.4 \cdot 10^{-2}$	51
MBB	1.0	$3.6 \cdot 10^{-3}$	194
NEM	5.0	$1.4 \cdot 10^{-2}$	51
IAA	10.0	$4.8 \cdot 10^{-3}$	144
DTNB	10.0	$1.2 \cdot 10^{-3}$	578
MBTA	1.3	$8.5 \cdot 10^{-4}$	815
BACA	1.0	$5.8 \cdot 10^{-4}$	1195
MPTA	1.3	$3.3 \cdot 10^{-5}$	>21000

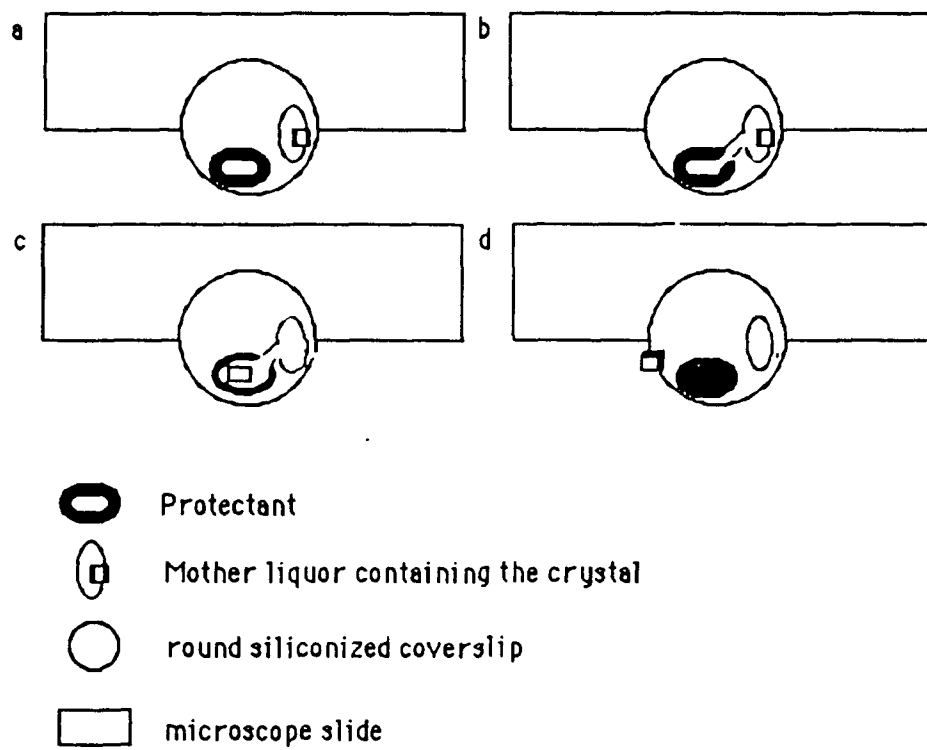
Abbreviations: BACA, bromoacetylcholamine; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); IAA, iodoacetamide; MBB, monobromobimane; MBTA, 4-(*N*-maleimido)- $\alpha$ -benzyltrimethylammonium iodide; MPTA, 4-(*N*-maleimido)phenyltrimethylammonium iodide; NEM, *N*-ethylmaleimide; PCMS, *p*-chloromercuriphenylsulfonic acid.

**TABLE 4.**      *Effects of cholinergic ligands on inactivation of AChE by NEM and PCMS*

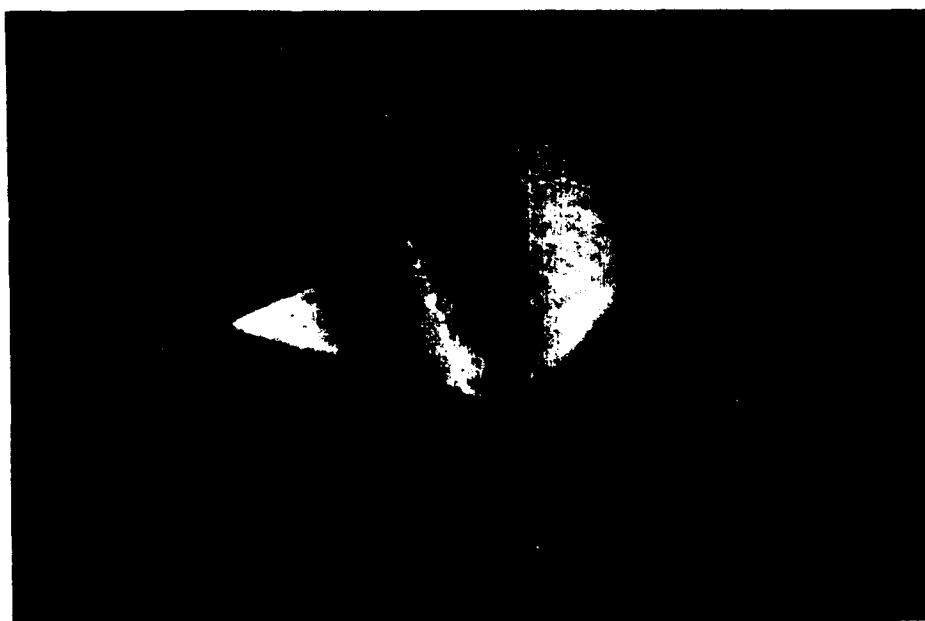
Concentrations of NEM and PCMS were 5 mM and 0.1 mM, respectively.

Sulphydryl reagent	Cholinergic ligand	Ligand concn. (M)	$k_i$ (min <sup>-1</sup> )	$t_{1/2}$ (min)
NEM	<i>N</i> -MAC	10 <sup>-5</sup>	1.4·10 <sup>-2</sup>	51
NEM	edrophonium	10 <sup>-5</sup>	1.6·10 <sup>-3</sup>	444
NEM	propidium	10 <sup>-6</sup>	2.9·10 <sup>-3</sup>	242
NEM	propidium	10 <sup>-4</sup>	full protection	
NEM	gallamine	10 <sup>-4</sup>	full protection	
PCMS	edrophonium	10 <sup>-5</sup>	4.3·10 <sup>-3</sup>	171
PCMS	gallamine	10 <sup>-4</sup>	5.1·10 <sup>-3</sup>	136

Abbreviations: NEM, *N*-ethylmaleimide; *N*-MAC, *N*-methylacridinium iodide; PCMS, *p*-chloromercuriphenylsulfonic acid.

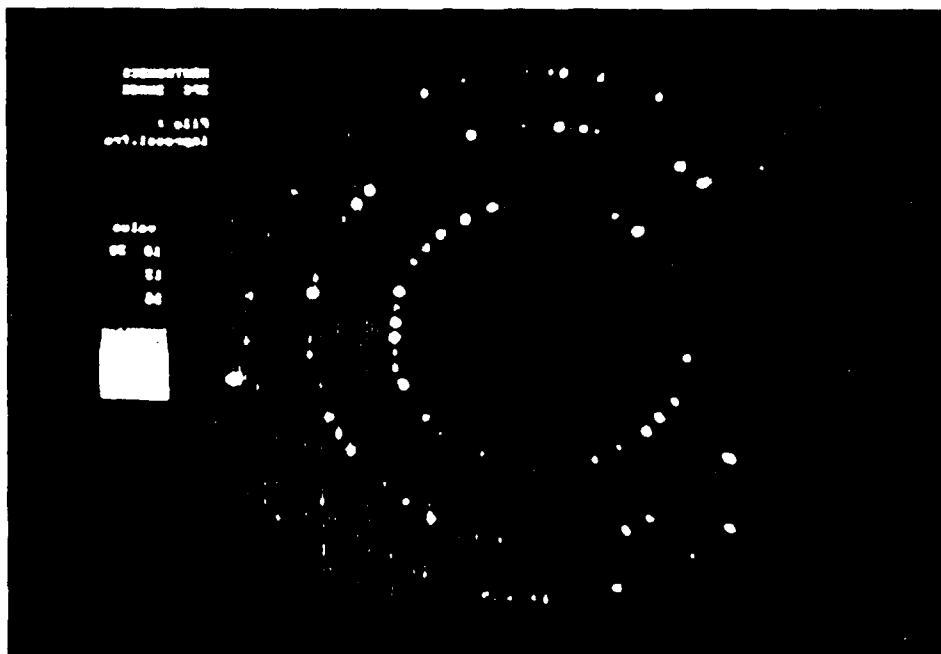


**Figure 1.** Crystal transfer from mother liquor to a cryoprotectant.

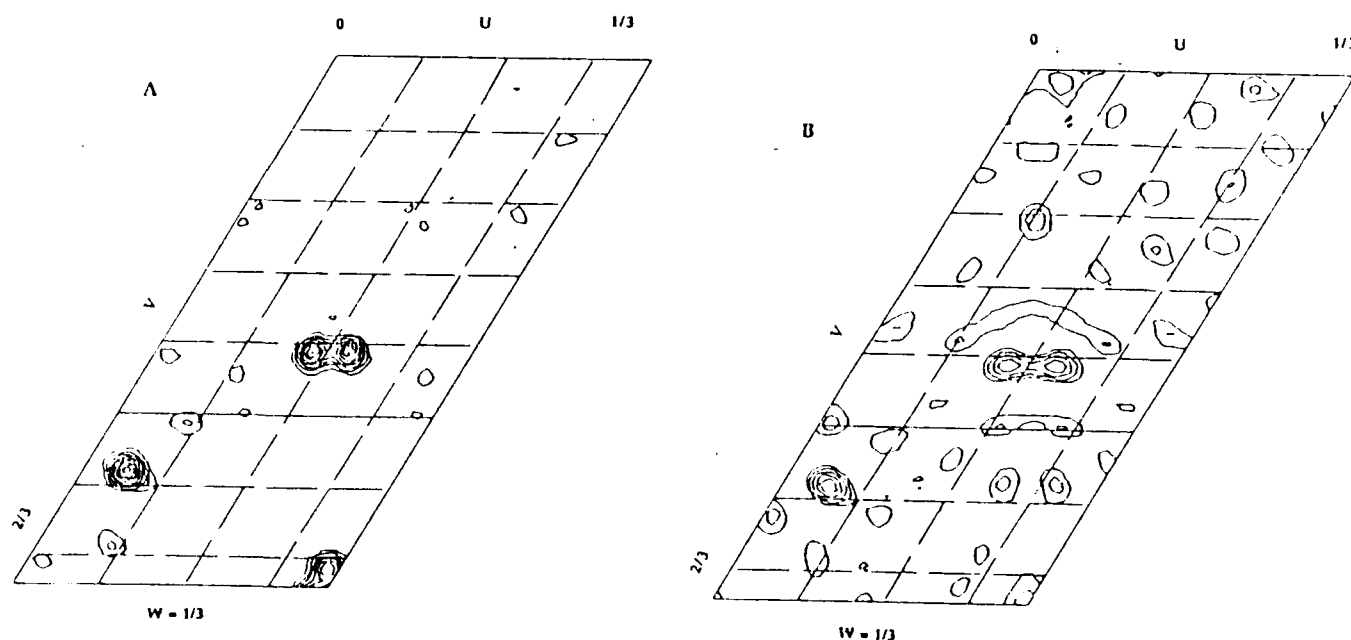


**Figure 2.** Crystals of AChE from *Torpedo californica* obtained by precipitation from concentrated ammonium sulfate. The crystals shown are from 0.6 to 1.0 mm in their longest dimension.

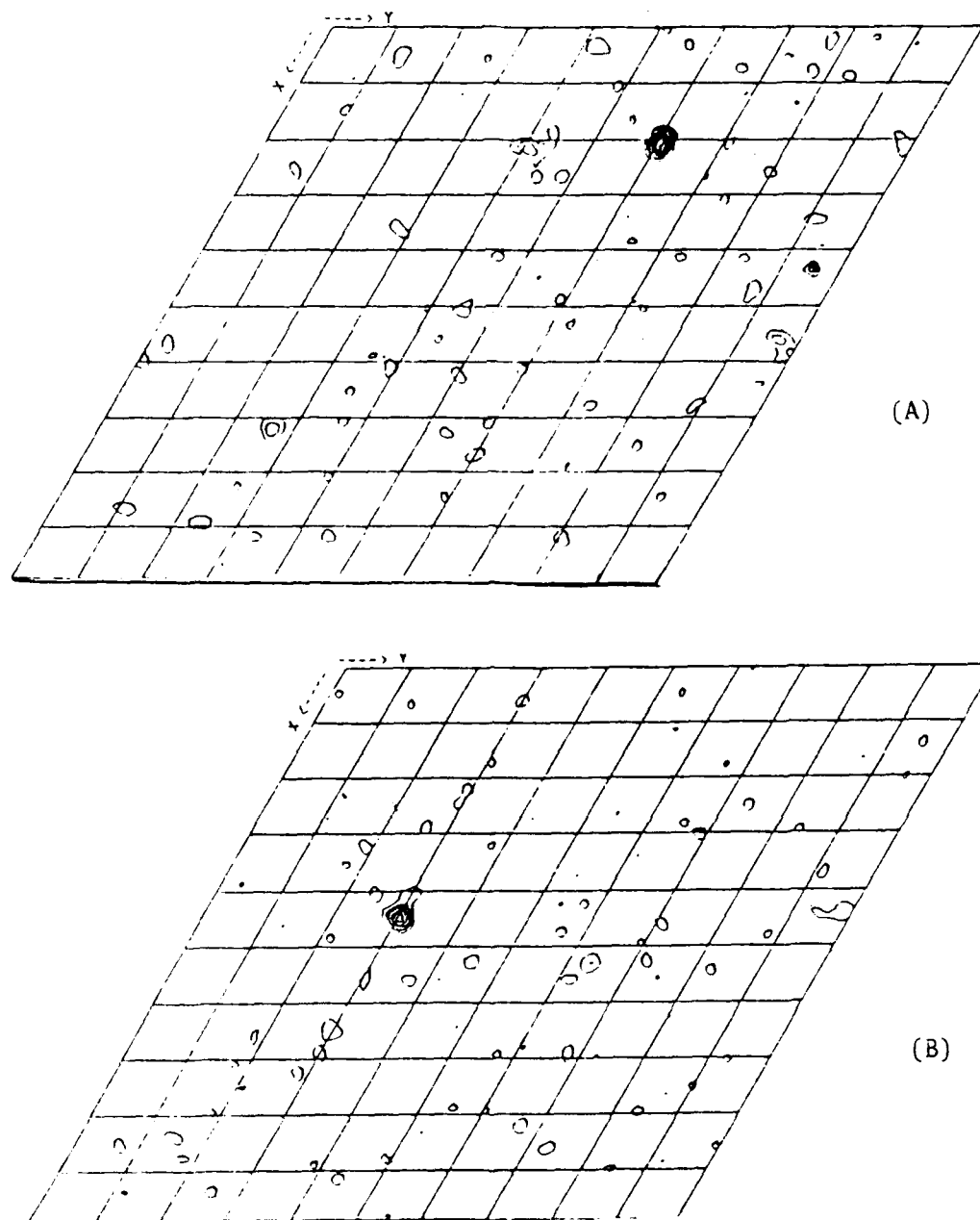




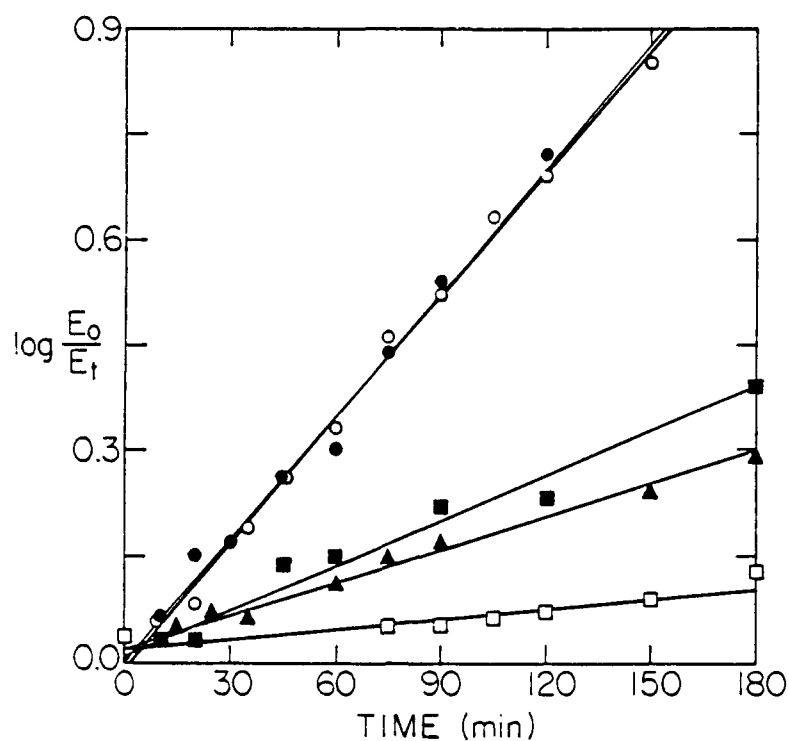
**Figure 3.** A single frame image obtained for a native crystal of *Torpedo* AChE on a Siemens/Xentronics area detector at room temperature. Exposure time, 120 sec; oscillation, 0.25°; crystal-to-detector distance, 12 cm;  $2\theta=10^\circ$ .



**Figure 4.** Harker section at  $w = 1/3$  of the difference Patterson (A) and the anomalous Patterson (B), calculated at resolutions of 3.5 and 5 Å, respectively, for the  $\text{UO}_2(\text{NO}_3)_2$  derivative of *Torpedo* AChE. The sections are contoured from  $1\sigma$ , in equal intervals of  $1\sigma$ , where  $\sigma$  is the standard deviation from the average density of the map.



**Figure 5.** Difference Fourier sections for the  $\text{HgAc}_2$  derivative of *Torpedo* AChE phased on the  $\text{UO}_2(\text{NO}_3)_2$  derivative. (A) Section at  $z = 0.08$ ; (B) Section at  $z = 0.02$ . The sections are contoured from  $2\sigma$ , in equal intervals of  $2\sigma$ , where  $\sigma$  is the standard deviation from the average density of the map.



**Figure 6.** Kinetics of inhibition of *Torpedo* AChE by sulfhydryl agents. Inhibition was performed in 50 mM sodium phosphate, pH 7.0, at room temperature, using  $10^{-7}$  M enzyme active sties. In the pseudo-first-order plots,  $E_0$  denotes activity prior to addition of inhibitor,  $E_t$  denotes activity at time t: o - o, 0.1 mM PCMS; • - •, 5 mM NEM; ■ - ■, 10 mM IAA; ▲ - ▲, 1 mM MBB; □ - □, 10 mM DTNB. Abbreviations: DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); IAA, iodoacetamide; MBB, monobromobimane; NEM, *N*-ethylmaleimide; PCMS, *p*-chloromercuriphenylsulfonic acid.

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